Supplemental Material, Figure 1

Materials and Methods

Reagents and antibodies. Microcystin-LR was purchased from Alexis (Lausen, Switzerland). Fetal calf serum was obtained from Cancera International (Canada). Lactacystin was purchased from Peptide Institute Inc. (Osaka, Japan). Minimum essential medium (MEM), a protease inhibitor cocktail, and pifithrin-α were purchased from Sigma (St. Louis, MO). The polyclonal rabbit anti-OATP1B3 antibody, SKT, was generated as previously described (König et al., 2000). Polyclonal rabbit antibodies against phospho-p53 (Ser^{6}, Ser^{9}, Ser^{15}, Ser^{20}, Ser^{37}, Ser^{46}, and Ser^{392}), phospho-PDK1 (Ser^{241}), phospho-Akt (Thr^{308}/Ser^{473}), phospho-GSK-3β (Ser^{9}), and phosphorylation state independent Akt were purchased from Cell Signaling Technology (Danvers, MA). Monoclonal mouse antibodies against GSK-3β and β-catenin were purchased from BD Biosciences (San Jose, CA). Monoclonal mouse antibody and agarose-conjugated antibody against p53 and polyclonal goat antibodies against Lamin B and β-actin were purchased from Santa Cruz (Santa Cruz, CA). Monoclonal mouse antibody against α-Tubulin was purchased from CALBIOCHEM (Darmstadt, Germany). Secondary antibodies against mouse IgG and rabbit IgG were purchased from Amersham (Buckinghamshire, United Kingdom), and against goat IgG was purchased from Jackson ImmunoResearch Laboratories (West Grove, PA). The GeneSuppressor™ System, containing pSuppressorNeo p53 and a pSuppressorNeo control plasmid, was purchased from IMGENEX Corporation (San Diego, CA). Lipofectamine™ 2000 was purchased from Invitrogen (Carlsbad, CA).

Cell culture. Wild-type p53-expressing human embryonic kidney cells, HEK293 cells, were stably transfected with the plasmids pcDNA3.1-*SLCO1B3* (HEK293-OATP1B3 cells) or pcDNA3.1-control vector (HEK293-CV cells), which were previously generated (König et al. 2000; Letschert et al. 2004). Cells were cultured in MEM supplemented with 10% fetal calf serum, 100 units/ml penicillin, 100 μg/ml streptomycin (MEM-10% FCS) and 400 μg/ml G418 at 37˚C, 100% humidity, and 5% CO_2.

Immunoblot Analysis. Crude membrane fractions or cell lysates were prepared from cultured transfected cells and were analyzed by immunoblotting as previously described (Komatsu et al. 2000, 2007). Proteins were separated by SDS/PAGE under reducing conditions. Samples were incubated for 5 min at 95˚C, alternatively in the case of OATP1B3, for 30 min at 37˚C to reduce aggregation of OATP1B3, prior to electrophoresis. Transfer to polyvinylidene difluoride (PVDF) membranes was

performed electrophoretically for 30 min at 15 V (constant voltage) using a semi-dry blotting system (Bio-Rad, Richmond, CA). The membranes were blocked with 5% BSA in TTBS (10 mM Tris-Cl, pH 8.0, 0.35 M NaCl, 0.05% Tween 20) for 1 hr at room temperature and were then incubated overnight at 4˚C with antibodies against human OATP1B3 (SKT), p53, phospho-p53, p21, phospho-PDK1, Akt, phospho-Akt, GSK-3β, phospho-GSK-3β, α-tubulin or β-actin. The membranes were washed three times with TTBS and were then incubated for 60 min with the appropriate horseradish peroxidase-conjugated secondary antibodies for detection of the protein of interest. Phosphoproteins were detected using Can Get Signal for IB (Toyobo, Osaka, Japan). The PVDF membranes were rinsed twice, washed four times for 5 min each time with TTBS, and were then evenly covered with the ECL Western blotting detection reagents (GE Healthcare, Buckinghamshire, UK) for 1 min. The membranes were immediately exposed to x-ray film (Fujifilm, Tokyo, Japan) in a film cassette at room temperature for various periods.

Immunoprecipitation. After incubation with microcystin-LR, cells were lysed with Lysis buffer (10 mM Tris-HCl, pH 8.0, 10 mM KCl, 1 mM EDTA, 1% Nonidet P-40, 1% protein inhibitor cocktail and 1 mM NaF). Following homogenization, the lysates were centrifuged at 10,000 x g for 30 min at 4˚C. One ml of the cell lysates was incubated overnight at 4˚C with 5 μl of agarose-conjugated anti-p53 antibody. The pellet was washed four times with Lysis buffer and was then suspended in SDS-polyacrylamide gel Laemmli sample buffer. Phosphorylation of p53 at Ser^{6}, Ser^{9}, Ser^{15}, Ser^{20}, Ser^{37}, Ser^{46}, and Ser^{392} in the same samples was analyzed after SDS/PAGE and immunoblotting with the respective phospho-p53 antibodies. After stripping with stripping buffer (0.5 M Tris-Cl, pH 6.8 containing 1% 2-ME) for 30 min at 50˚C, the blots were reprobed with an anti-p53 antibody.

Cytotoxicity studies. Colorimetric assay using the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) was used to assess the sensitivity of the cells to microcystin-LR *in vitro* as described (Komatsu et al., 2000). Exponentially growing cells were trypsinized and harvested, and equal numbers (1.6×10^{4}) of cells in 180 μl of MEM-10% FCS were inoculated into each well of a 96-well microplate. After incubation overnight, 20 μl of microcystin-LR solutions were added to the cultures, and then they were incubated for 3 days. Thereafter, 50 μl of 1 mg/ml MTT solution was added to each well, and the plates were incubated for 3 hr at 37˚C in a CO_2 incubator. After aspirating the culture medium, the resulting formazan was dissolved with dimethylsulfoxide. Plates were placed on a shaker for 5 min and read immediately at 570 nm with a microplate reader, MPR-A4i (Tosoh, Tokyo, Japan),

and cell viability was determined. The IC_{50}-value was determined as the concentration of microcystin-LR that reduced the viability of the cells to 50% of that in control medium.

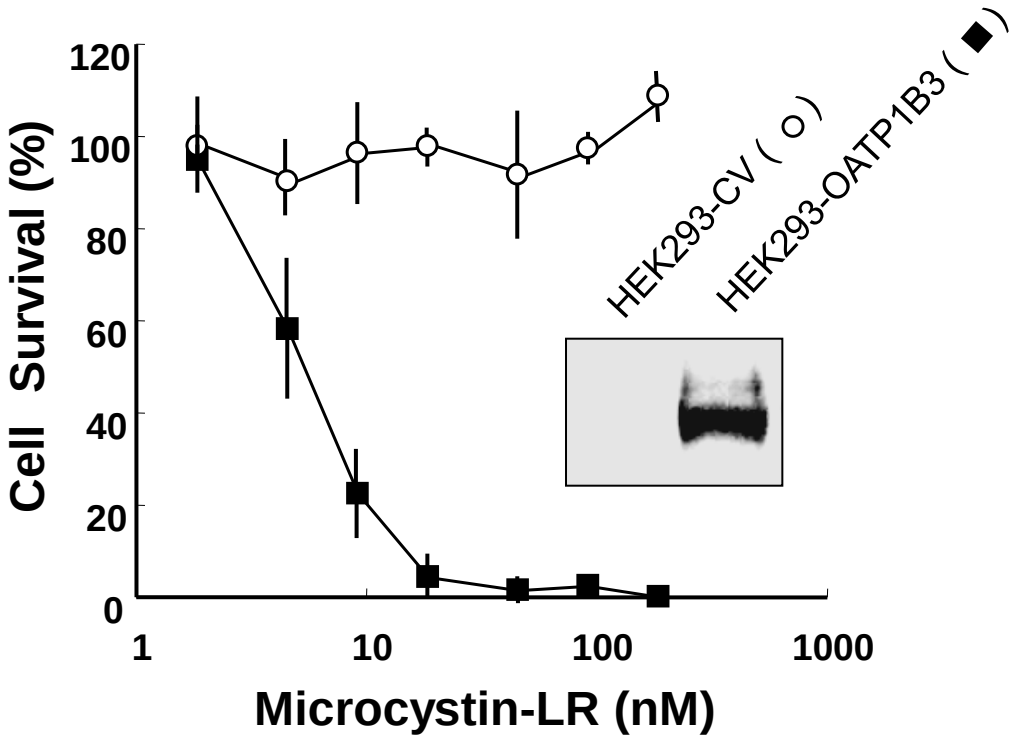
For inhibition studies, cells in 170 µl of MEM-10% FCS were pre-treated with 10 µl of pifithrin-α (30 µM) and were then incubated for 3 days with 20 µl of a microcystin-LR solution.

Result

Cytotoxicity of microcystin-LR. We first examined the effect of stable transfection of OATP1B3 on the cytotoxic effect of microcystin-LR using control, HEK293-CV and HEK293-OATP1B3 cells. HEK293-OATP1B3 cells were highly sensitive to microcystin-LR (IC_{50}: 6.5 nM) in an MTT cell viability assay. In contrast, HEK293-CV cells were resistant to microcystin-LR up to a concentration of 200 nM (Supplemental Figure 1). The expression level of OATP1B3 was negligible in the HEK293-CV cells, while its expression was readily detectable by immunoblotting in HEK293-OATP1B3 cells (Supplemental Figure 1).

Figure legend

Supplemental Figure 1. Effect of OATP1B3 on the cytotoxicity of microcystin-LR. HEK293-CV and HEK293-OATP1B3 cells were incubated with the indicated concentrations of microcystin-LR for 3 days following which cell viability was measured using the MTT assay. Cell survival is expressed as a percentage of the viable cell number prior to exposure. The data represent the mean values ± SD of three independent experiments each performed in triplicate. The inset panel shows the expression levels of OATP1B3 in HEK293-CV and HEK293-OATP1B3 cells analyzed by immunoblotting.



Supplemental Figure 1